

## Development of a Multiplex Real-Time PCR Assay with an Internal Amplification Control for the Detection of Total and Pathogenic *Vibrio parahaemolyticus* Bacteria in Oysters<sup>▽</sup>

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*Vibrio parahaemolyticus* is an estuarine bacterium that is the leading cause of shellfish-associated cases of bacterial gastroenteritis in the United States. Our laboratory developed a real-time multiplex PCR assay for the simultaneous detection of the thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), and thermostable-related hemolysin (*trh*) genes of *V. parahaemolyticus*. The *tlh* gene is a species-specific marker, while the *tdh* and *trh* genes are pathogenicity markers. An internal amplification control (IAC) was incorporated to ensure PCR integrity and eliminate false-negative reporting. The assay was tested for specificity against >150 strains representing eight bacterial species. Only *V. parahaemolyticus* strains possessing the appropriate target genes generated a fluorescent signal, except for a late *tdh* signal generated by three strains of *V. hollisae*. The multiplex assay detected <10 CFU/reaction of pathogenic *V. parahaemolyticus* in the presence of >10<sup>4</sup> CFU/reaction of total *V. parahaemolyticus* bacteria. The real-time PCR assay was utilized with a most-probable-number format, and its results were compared to standard *V. parahaemolyticus* isolation methodology during an environmental survey of Alaskan oysters. The IAC was occasionally inhibited by the oyster matrix, and this usually corresponded to negative results for *V. parahaemolyticus* targets. *V. parahaemolyticus* *tlh*, *tdh*, and *trh* were detected in 44, 44, and 52% of the oyster samples, respectively. *V. parahaemolyticus* was isolated from 33% of the samples, and *tdh*<sup>+</sup> and *trh*<sup>+</sup> strains were isolated from 19 and 26%, respectively. These results demonstrate the utility of the real-time PCR assay in environmental surveys and its possible application to outbreak investigations for the detection of total and pathogenic *V. parahaemolyticus*.

*Vibrio parahaemolyticus* is found worldwide in estuarine waters and is the leading cause of gastroenteritis from seafood in the United States, with most infections resulting from the consumption of raw or mishandled seafood (2, 29). The *tlh* (thermolabile hemolysin) gene is a species-specific marker for *V. parahaemolyticus* (42, 26), while the *tdh* (thermostable direct hemolysin) (33, 34) and *trh* (thermostable-related hemolysin) (35) genes are pathogenicity markers for *V. parahaemolyticus*. Pathogenic *V. parahaemolyticus* bacteria in the environment and food samples typically comprise 0.3 to 3% of the total *V. parahaemolyticus* population (4, 8, 20, 22, 43). DNA-based assays targeting these genes were developed for the detection and enumeration of *V. parahaemolyticus* bacteria (7, 10, 21, 26, 27, 30, 38, 44). However, the ability of the PCR assays to detect low levels of pathogenic *V. parahaemolyticus* bacteria in the presence of a high background of nonpathogenic *V. parahaemolyticus* bacteria was not reported by the authors (10, 30, 44). The preferential amplification of nonpathogenic members of the total *V. parahaemolyticus* population due to the much-

higher copy number of the target could preclude the detection of pathogenic strains present in lower numbers.

One of the risks associated with testing samples, especially food and environmental samples, by PCR is the occurrence of false negatives resulting from PCR inhibition (1, 18, 41, 45). While positive and negative controls are normally run with every PCR master mix to ensure the integrity of the reagents, PCR inhibition by the sample matrix can prevent the amplification of the target template, resulting in false-negative reporting (18, 39, 45). This is more of a concern with quantitative real-time PCR assays, as partial PCR inhibition can lead to inaccurate quantification results. Therefore, it is necessary to include an internal amplification control (IAC) in each individual reaction mixture to prevent the reporting of false negatives and to allow accurate adjustments to quantitative data (18). Previous works have utilized various methods of developing and using an IAC, including, but not limited to, housekeeping genes and synthetic plasmid constructs (15, 17, 23, 39, 40).

The goal of the current study was to develop a real-time PCR assay for the detection of total and pathogenic *V. parahaemolyticus* bacteria in oysters. While there are such assays published, we desired an assay which can detect pathogenic strains (*tdh* and/or *trh* positive) in a background of at least 1,000-fold more nonpathogenic *V. parahaemolyticus* bacteria. Additionally, we wanted to include an IAC to differentiate between negative samples and indeterminate

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results. To determine the applicability of this assay for the detection of *V. parahaemolyticus* in the environment, we examined oyster samples using a most-probable-number (MPN)-PCR format (24, 25, 30, 31, 41).

We describe a four-target real-time multiplex PCR assay developed on the SmartCycler II system from Cepheid (Sunnyvale, CA). This assay includes a novel exogenous IAC and is designed for the robust and simultaneous detection and quantification of total (*tlh*) and potentially pathogenic (*tdh* and/or *trh*) strains of *V. parahaemolyticus*. While the assay does have quantitative capability, directly analyzing oyster homogenate would give a limit of detection of only 500 CFU/ml (the assay can detect 1 CFU/2  $\mu$ l reaction template). As pathogenic *V. parahaemolyticus* densities are generally below this level, we utilized this assay in conjunction with an MPN enrichment during an environmental survey in Alaska.

#### MATERIALS AND METHODS

**Bacterial cultures and DNA template preparation.** Selected *V. parahaemolyticus* strains (FIHES98, a *tdh*-negative/*trh*-negative clinical isolate; TX2103, a *tdh*-positive/*trh*-negative clinical isolate; AQ4037, a *tdh*-negative/*trh*-positive clinical isolate; and F11-3A, a *tdh*-positive/*trh*-positive environmental isolate) used for the generation of standard curves were grown overnight at 35°C in tryptic soy broth (TSB; Difco, Sparks, MD). A 1:100 dilution of the overnight culture was made in fresh TSB and incubated for 4 to 6 h at 35°C with shaking (~150 rpm) to achieve a mid-exponential-phase culture. Dilutions were made of each exponential-phase culture in phosphate-buffered saline (PBS; 7.65 g NaCl, 0.724 g Na<sub>2</sub>HPO<sub>4</sub> [anhydrous], 0.21 g KH<sub>2</sub>PO<sub>4</sub> per liter of distilled water, pH 7.4). Crude cell lysates were prepared from 1-ml aliquots of each of these dilutions by boiling for 10 min in 1.5-ml microcentrifuge tubes (7). Additionally, dilutions were spread onto tryptic soy agar (TSA; Difco) plates prior to being boiled and incubated overnight at 35°C. The next day, colonies were counted to determine the CFU/ml for each preparation of boiled cells. The genomic DNA contained in the boiled lysates was used as a template for development of the real-time PCR assay. All additional strains utilized in this study were grown overnight at 35°C in TSB and boiled as described above; 2  $\mu$ l was used as a template in the real-time PCR.

**Design and evaluation of primers and fluorogenic probes for real-time PCR.** The complete nucleotide sequences (open reading frame regions only) for all reported variants of the *tlh*, *tdh*, and *trh* genes of *V. parahaemolyticus* were obtained from GenBank, aligned, and compared using Lasergene MegAlign software (clustal alignment and PAM250 distance tables) from DNASTAR (Madison, WI). Primer Express software from Applied Biosystems (Foster City, CA) was used to design oligonucleotide primers and TaqMan-style or TaqMan MGB fluorogenic probes targeting regions identified by the alignments as specific for each of these genes. Primer and probe sequences are listed in Table 1.

**PCR IAC.** A PCR IAC of novel design was incorporated into this assay (6; United States and international patents pending [http://appft1.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=%2Fnetacghtml%2FPTO%2Fsearch-bool.html&r=1&f=G&l=50&co1=AND&d=PG01&s1=blackstone&s2=vickery&OS=blackstone+AND+vickery&RS=blackstone+AND+vickery]). The IAC utilizes a linear synthetic exogenous DNA, specific oligonucleotide primers, and a fluorogenic probe (Table 1) that are incorporated into the PCR master mix and coamplified with the target sequences in each reaction. The sequence of the IAC DNA has no significant nucleotide identity to any known, naturally occurring, PCR-amplifiable nucleotide sequences reported in the NCBI database. For the present study, the IAC primer concentration was limited and the control DNA titrated to allow the detection of the IAC (cycle threshold [*C<sub>T</sub>*] values of ~20) under noninhibitory conditions without changing the amplification or detection characteristics of the other targets in the assay (Fig. 1). The IAC was applied in this manner to allow the detection of various amounts of inhibition.

**Real-time PCR amplification.** The real-time PCR cycling protocol, fluorescent detection parameters, and reaction mixture component concentrations were optimized for the multiplex detection and quantification of the *tlh*, *tdh*, and *trh* genes of *V. parahaemolyticus* and the IAC. The optimal conditions for PCR were run for a 25- $\mu$ l volume using the following reaction mixture components (final concentrations): 1 $\times$  PCR amplification buffer (Invitrogen, Carlsbad, CA), 5.0 mM MgCl<sub>2</sub> (Invitrogen), 400 nM of each of the deoxynucleoside triphosphates

TABLE 1. Real-time PCR primer and probe sequences utilized in the multiplex assay

Primer or probe	Sequence (5' to 3')	Modifications <sup>a</sup>
<i>tdh</i> forward	TCCCTTTTCCTGCCCCC	
<i>tdh</i> reverse	CGCTGCCATTGTATAGTCTT	
	TATC	
<i>tdh</i> probe	TGACATCCTACATGAC	5' FAM to 3'
	TGTG	MGBNFQ
<i>trh</i> forward	TTGCTTTTCAGTTTGCTATT	
	GGCT	
<i>trh</i> reverse	TGTTTACCGTCATATAGGC	
	GCTT	
<i>trh</i> probe	AGAAATACAACAATCAAAA	5' TET to 3'
	CTGA	MGBNFQ
<i>tlh</i> forward	ACTCAACACAAGAAGAGAT	
	CGACAA	
<i>tlh</i> reverse	GATGAGCGGTTGATGT	
	CCAA	
<i>tlh</i> probe	CGCTCGCGTTCACGAAA	5' TxRED to
	CCGT	3' BHQ2
IAC forward	GACATCGATATGGGTGCCG	
IAC reverse	CGAGACGATGCAGCCATTC	
IAC probe	TTCATGCGTCTCCCTGGTG	5' Cy5 to 3'
	AATGTG	BHQ2

<sup>a</sup> FAM, 6-carboxyfluorescein; MGBNFQ, minor groove binding nonfluorescent quencher; TET, tetrachlorofluorescein; TxRED, Texas Red; BHQ2, black hole quencher 2.

(Roche, Indianapolis, IN), 200 nM each of the *trh* and *tdh* forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 75 nM each of the *tlh* and IAC forward and reverse primers (Integrated DNA Technologies), 150 nM probe for *tlh* and IAC (Integrated DNA Technologies), 75 nM probe for *tdh* and *trh* (Applied Biosystems) (Table 1), and 2.25 U platinum *Taq* DNA polymerase (Invitrogen). The remainder of the reaction mixture volume consisted of a previously titrated concentration (2  $\mu$ l) of the IAC DNA, nuclease-free H<sub>2</sub>O, and a DNA template (2  $\mu$ l of boiled cells or enrichment).

Real-time PCR thermal cycling was conducted using a SmartCycler II system from Cepheid (Sunnyvale, CA). The optimal cycling parameters consisted of a 95°C hold for 60 s for the initial denaturation and activation of the hot-start *Taq* polymerase, followed by 45 cycles of amplification, with each cycle consisting of denaturation at 95°C for 5 s and a combined primer annealing/extension step at 59°C for 45 s. Fluorescence was read at the end of each amplification cycle by using a fluorescein isothiocyanate-25 dye set. The default software analysis settings were used, except that the manual threshold fluorescence units setting was adjusted to 15 fluorescence units to provide the greatest sensitivity without the detection of false positives due to fluorescence background "noise." Positive controls, consisting of a *V. parahaemolyticus* strain possessing all three target genes, and a negative control (nuclease-free H<sub>2</sub>O added as a template) were prepared for each PCR master mix.

**Generation of standard curves.** For the determination of the assay limit of detection, dynamic range, and quantitative capabilities, a *V. parahaemolyticus* strain (F11-3A) possessing all three target genes (*tlh*, *tdh*, and *trh*) was used to generate standard curves for each target during multiplex real-time PCR amplification. Tenfold serial dilutions in PBS were prepared from boiled cells of 4- to 6-h (mid-exponential-phase) cultures from which the CFU/ml values were obtained (see above). The assay was run in triplicate for each dilution, using 2  $\mu$ l of template per reaction. The amplification efficiency was calculated by using the formula  $E = 10^{(-1/\text{slope})} - 1$ , where *E* is efficiency. The efficiency was converted into a percentage by multiplying by 100. The slope was determined by plotting the *C<sub>T</sub>* values against the log CFU/reaction; the linear regression was calculated by using Sigma Plot software (Systat, San Jose, CA).

**Specificity and robustness testing.** Using boiled cells as a source of template DNA, the assay was tested for specificity against a panel of 153 bacterial isolates, including *Vibrio alginolyticus*, *V. cholerae*, *V. fluvialis*, *V. hollisae*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus*. The *V. parahaemolyticus* strains included 64 clinical isolates (25 from five Asian countries, 16 from Wash-

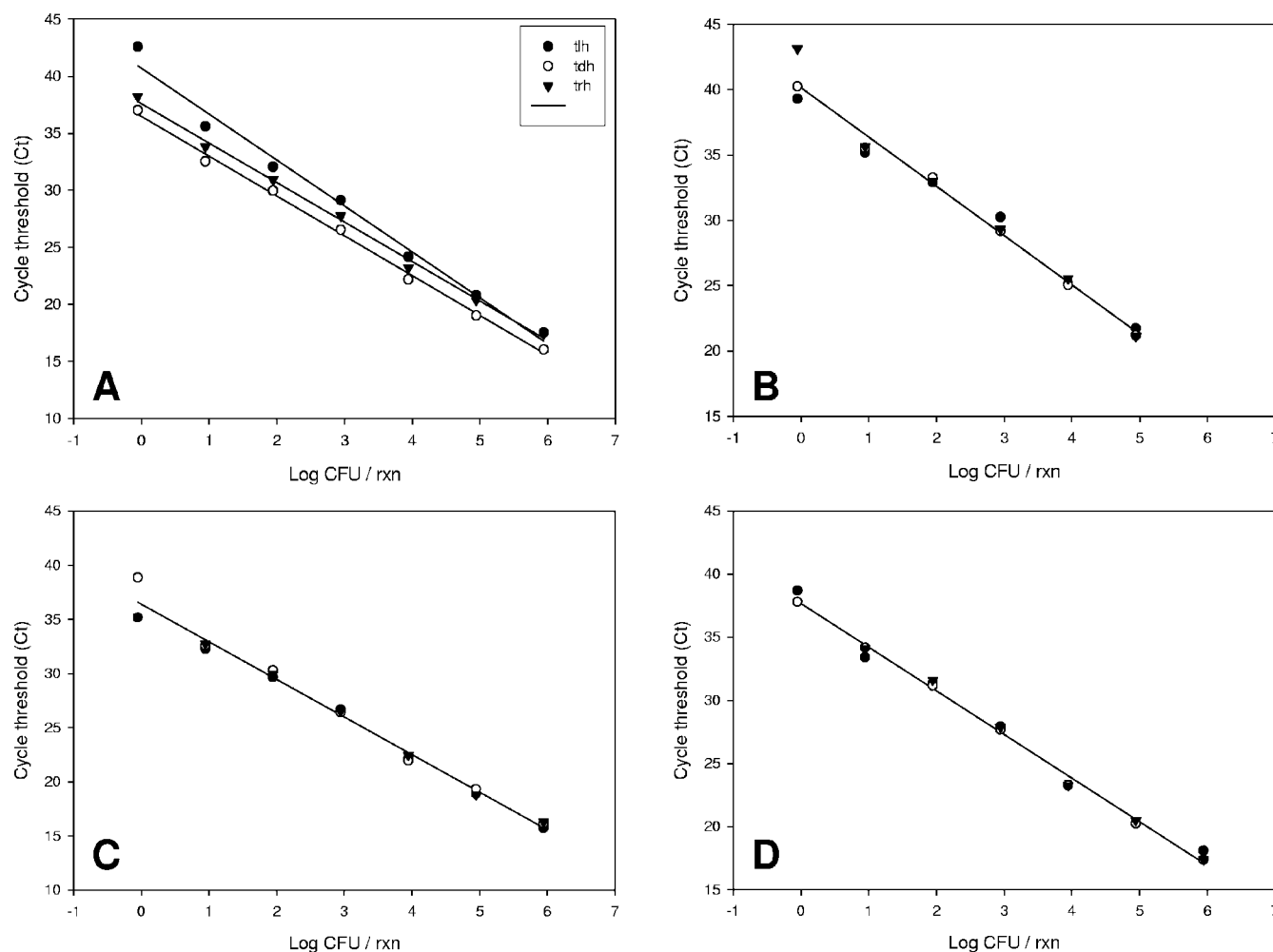


FIG. 1. Standard curves of three target genes in multiplex and simplex PCR. (A) Standard curve for the multiplex assay as described in Materials and Methods; the  $r^2$  value was 0.99 for the standard curve of each target. (B, C, and D) Standard curves for each of the targets when run as a single target with the same reaction conditions as described in Materials and Methods. Results for the *tlh*, *tdh*, and *trh* targets are shown in panels B, C, and D, with  $r^2$  values of 0.98, 0.99, and 0.99, respectively. Each standard curve was run in triplicate; each replicate is plotted. *V. parahaemolyticus* with all three target genes from an exponential-phase culture was diluted in PBS, boiled, and used as a template for all experiments, with concentrations of  $0.9 \times 10^5$  to  $9 \times 10^5$  CFU/reaction, except that the *tlh* simplex assay was tested with  $0.9 \times 10^4$  to  $9 \times 10^4$  CFU/reaction. rxn, reaction.

ington, 11 from Texas, 6 from New York, 5 from California, and 1 from Connecticut), 36 environmental isolates (13 from Alabama, 12 from Alaska, 10 from Washington, and 1 from New York), 7 food isolates from the Pacific Northwest, and 10 isolates of unknown source. Additionally, various concentrations of pathogenic strains were tested in combination with higher concentrations of nonpathogenic strains to determine the ability of the assay to detect low copy numbers of the *tdh* and *trh* genes against a very high background of the *tlh* gene, as might be expected for environmental samples.

**Environmental studies. (i) Sample collection.** Twenty-seven oyster samples were collected at various locations in Prince William Sound and other coastal areas of Alaska from August 14 to September 11, 2004. Oysters (10 to 12 per sample) were collected from cages suspended from rafts and transported on bagged ice to the analyzing laboratory (Alaska Department of Environmental Conservation, Palmer, AK). Analysis was conducted within 24 h of collection.

**(ii) Sample preparation.** Oysters were scrubbed and shucked according to American Public Health Association guidelines (3). The entire shell contents (animal and liquor) were emptied into a sterile blender and homogenized at high speed for 90 s. A three-tube MPN method was done as described in the FDA Bacteriological Analytical Manual (11), with slight modifications. Briefly, 10 g of homogenate was added to 90 ml of alkaline peptone water (APW; 1% peptone, 1% NaCl, pH  $8.5 \pm 0.2$ ) in each of three separate

bottles. One gram of homogenate was added to 10 ml of APW in each of three separate tubes, and 1 g was added to 9 ml of PBS for dilutions. A 10-fold dilution series was done in PBS, and 1 ml of each dilution was added to 10 ml of APW in each of three tubes. All tubes were incubated overnight at 35°C.

**(iii) Culture isolation.** After overnight incubation, the top 1 cm of growth from all APW tubes was streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS; Difco, Sparks, MD) plates. The TCBS plates were incubated overnight at  $35 \pm 2^\circ\text{C}$  and suspect colonies were streaked for purification onto nonselective media (TSA with an additional 2% NaCl added). Purified isolates were subjected to further biochemical identification that included oxidase, arginine glucose slant, motility test medium (MTM), urea broth, and salt tolerance. Suspect *V. parahaemolyticus* isolates were confirmed by using an API 20E assay (bioMérieux, Inc., Durham, NC) and sent to the FDA Gulf Coast Seafood Laboratory for characterization by alkaline phosphatase (AP) gene probe hybridization. Each isolate was inoculated into 100  $\mu\text{l}$  of APW in a 96-well plate and incubated overnight. Growth was transferred to a  $\text{T}_1\text{N}_3$  (1% tryptone, 3% sodium chloride, 2% agar) plate with a 48-prong replicator (Boeckel, Feasterville, PA). After overnight incubation, colony lifts were done as previously described (11, 36) and isolates were identified using AP-*tlh*, AP-*tdh*, and AP-*trh* probes (11, 36). Any tube that produced at least one confirmed *V. parahaemolyticus* (*tlh*-positive) isolate was scored positive for

TABLE 2. Strains tested for assay specificity

Species (no. of isolates)	Gene target		
	<i>tlh</i>	<i>tdh</i>	<i>trh</i>
<i>V. parahaemolyticus</i> (5)	+	—	—
<i>V. parahaemolyticus</i> (38)	+	+	—
<i>V. parahaemolyticus</i> (13)	+	—	+
<i>V. parahaemolyticus</i> (61)	+	+	+
<i>V. cholerae</i> (8)	—	—	—
<i>V. mimicus</i> (6)	—	—	—
<i>V. fluvialis</i> (6)	—	—	—
<i>V. hollisae</i> (1)	—	—	—
<i>V. hollisae</i> (3) <sup>a</sup>	—	+	—
<i>V. metschnikovii</i> (1)	—	—	—
<i>V. vulnificus</i> (10)	—	—	—
<i>V. alginolyticus</i> (1)	—	—	—

<sup>a</sup>  $C_T$  values for *tdh* were 33.28, 35.22, and 36.23; *V. parahaemolyticus* strains generated  $C_T$  values of 15.54 to 18.72 for the *tdh* gene at similar concentrations.

total *V. parahaemolyticus* bacteria. Any tube that produced at least one isolate determined to be *tdh*- or *trh*-positive by the AP probe was scored positive for the respective gene.

(iv) **Real-time PCR analysis.** After incubation, a 1-ml portion from each tube of APW was removed and boiled for 10 min. They were then stored frozen at  $-20^{\circ}\text{C}$  until the completion of the study (~6 weeks). All tubes were shipped to the FDA Gulf Coast Seafood Laboratory for analysis of *tlh*, *tdh*, and *trh* genes by real-time PCR as described above.

## RESULTS

The real-time PCR assay was tested for specificity against a panel of 117 *V. parahaemolyticus* isolates with various combinations of gene targets (Table 2). Isolates from clinical, environmental, and food sources from a variety of geographical locations were tested. All *V. parahaemolyticus* strains examined gave the expected results based on testing with previously published methods (5, 37). Additionally, 8 *V. cholerae*, 6 *V. mimicus*, 6 *V. fluvialis*, 4 *V. hollisae*, 1 *V. metschnikovii*, and 10 *V. vulnificus* strains and 1 *V. alginolyticus* strain were tested (Table 2). Three of the four *V. hollisae* strains were positive for *tdh*. The *V. hollisae* strains generated  $C_T$  values of 35.22, 33.28, and

36.23. The *V. parahaemolyticus* strains generated  $C_T$  values for all three targets ranging from 15.54 to 22.62. All other strains were negative for the three gene targets.

The quantitative ability of the multiplex assay is demonstrated in Fig. 1A, where all three targets show similar reaction efficiencies and limits of detection. The standard curves demonstrate that each target can be detected down to 1 CFU/reaction in a multiplex assay. The amplification efficiencies in the multiplex assay were 82, 94, and 95% for *tlh*, *tdh*, and *trh* targets, respectively, indicating the ability of this assay to effectively quantify each target. Panels B, C, and D in Fig. 1 show standard curves for the *tlh* target, *tdh* target, and *trh* target, respectively, when run as individual targets. The amplification efficiency of each target is not significantly different (<2% difference) in the multiplex assay than when run individually. However, the efficiency of the *tlh* reaction is increased to 91% when primer limiting is not employed (data not shown).

The assay was optimized for the detection of low numbers (<10 CFU/reaction) of pathogenic (*tdh*-positive and/or *trh*-positive) *V. parahaemolyticus* bacteria in the presence of high numbers ( $>10^4$  CFU/reaction) of total *V. parahaemolyticus* bacteria by primer limiting the *tlh* reaction. Instead of utilizing the optimal final concentration of each *tlh* primer (200 nM), the multiplex assay uses only 75 nM of each *tlh* primer. Table 3 shows reliable detection of 4 CFU/reaction of *tdh*-positive/*trh*-positive *V. parahaemolyticus* in a background of  $7 \times 10^4$  CFU/reaction of nonpathogenic *V. parahaemolyticus* (1:15,000); in contrast, at least  $4 \times 10^2$  pathogenic *V. parahaemolyticus* bacteria are needed for the detection in the same background of nonpathogenic strains (1:150) when primer limiting of the *tlh* reaction is not employed.

The IAC incorporated into the assay was designed to give a  $C_T$  value of 20 under noninhibitory conditions, regardless of the number of *V. parahaemolyticus* targets present. In the runs used to generate the pure-culture standard curves (Fig. 1), the average  $C_T$  value for the IAC was  $19.99 \pm 0.27$  (Fig. 2A). Table 3 demonstrates the reproducibility of the IAC.

The IAC was also designed to shift in the presence of PCR

TABLE 3. Effect of *tlh* primer limiting on the detection of pathogenic *V. parahaemolyticus* bacteria in low ratio to nonpathogenic *V. parahaemolyticus* bacteria

Final concn of <i>tlh</i> primer and CFU/reaction of target gene:		Ratio of <i>tdh/trh</i> to <i>tlh</i>	$C_T \pm \text{SD}^a$ for:			
<i>tdh/trh</i>	<i>tlh</i>		<i>tdh</i>	<i>trh</i>	<i>tlh</i>	IAC
75 nM						
4,400	70,000	1:15	23.62 ± 0.33	24.41 ± 0.87	20.76 ± 0.47	20.31 ± 0.21
440	70,000	1:150	29.44 ± 0.17	30.83 ± 0.43	21.01 ± 0.14	20.34 ± 0.43
44	70,000	1:1,500	33.31 ± 0.98	33.82 ± 0.13	21.19 ± 0.08	20.41 ± 0.23
4.4	70,000	1:15,000	36.36 ± 0.49	37.47 ± 1.44 <sup>b</sup>	21.06 ± 0.22	20.18 ± 0.05
0.44	70,000	1:150,000	0.00 ± 0.00	0.00 ± 0.00	21.19 ± 0.02	19.97 ± 0.24
200 nM						
4,400	70,000	1:15	24.31 ± 1.79	24.40 ± 0.88	21.13 ± 0.08	20.29 ± 0.17
440	70,000	1:150	40.40 ± 0.77 <sup>b</sup>	0.00 ± 0.00	21.14 ± 0.07	20.37 ± 0.23
44	70,000	1:1,500	0.00 ± 0.00	0.00 ± 0.00	21.23 ± 0.11	20.23 ± 0.10
4.4	70,000	1:15,000	0.00 ± 0.00	0.00 ± 0.00	21.23 ± 0.02	20.02 ± 0.22
0.44	70,000	1:150,000	0.00 ± 0.00	0.00 ± 0.00	21.16 ± 0.08	19.90 ± 0.08

<sup>a</sup> SD is the standard deviation of the  $C_T$  values based on the results for three replicates.

<sup>b</sup> Only two replicates generated a  $C_T$  value; the SD was calculated excluding the negative replicate.

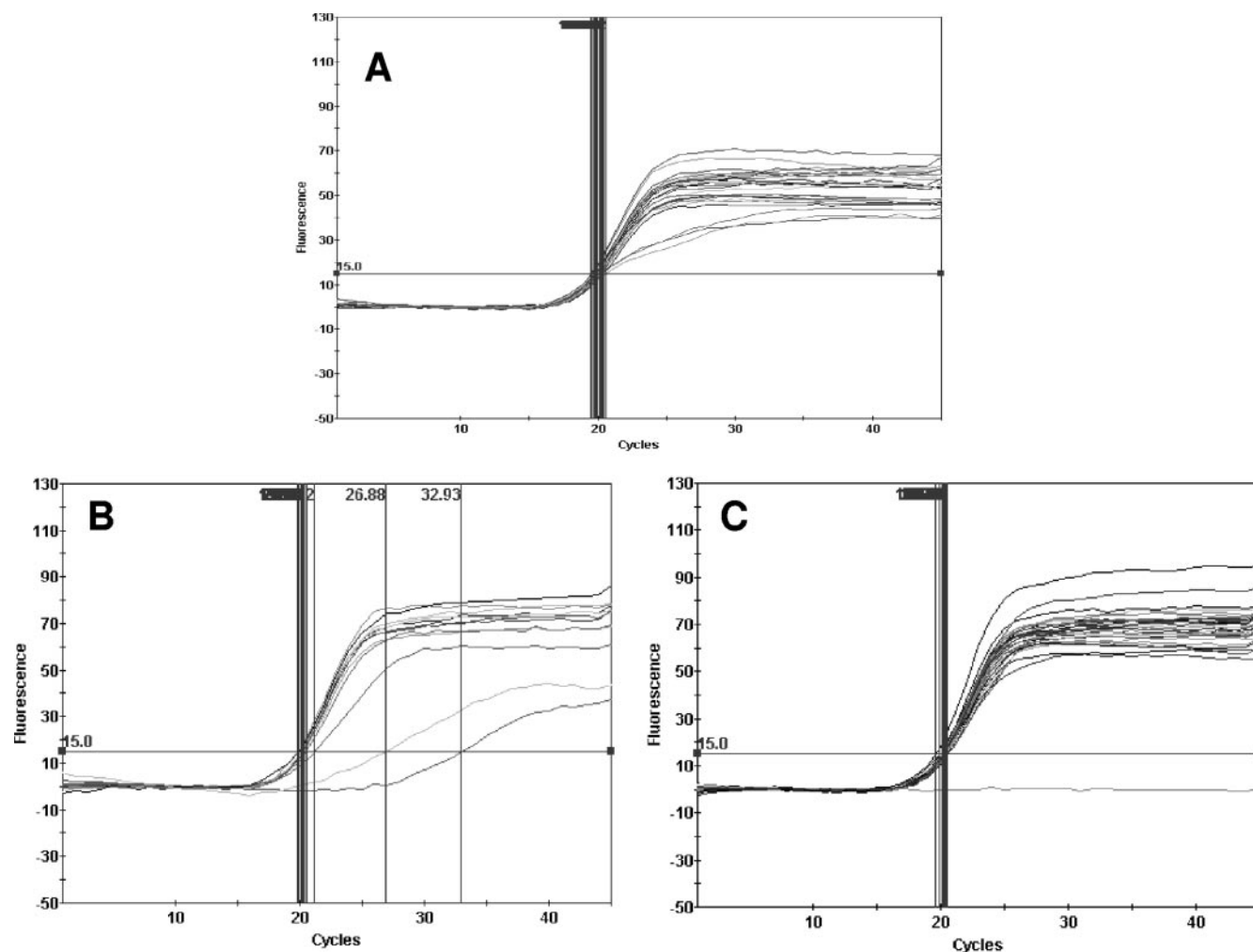


FIG. 2. Reporting of the IAC without and with sample matrix inhibition. (A) Consistent reporting ( $C_T$  of 19 to 21) of the IAC in the presence of  $0.9 \times 10^5$  to  $9 \times 10^5$  CFU/reaction of *V. parahaemolyticus* targets is demonstrated. (B and C) Shifts and complete inhibition of the IAC, respectively, in some Alaskan oyster samples are shown. The large number of reactions without any shift emphasizes the overall lack of inhibition seen in the Alaskan oyster samples.

inhibition from sample matrices, to aid in accurate reporting of results. Figures 2B and C demonstrate a shift and complete inhibition, respectively, of the IAC in oyster samples. In Fig. 2B, various degrees of inhibition are seen in two of the samples; *V. parahaemolyticus* targets were not detected in either of these tubes. Complete inhibition of the IAC is illustrated in Fig. 2C; no *V. parahaemolyticus* targets were detected in this sample. Overall, of the Alaskan oyster samples (306 MPN tubes), four tubes from four different samples had complete inhibition of the IAC; three tubes from three different samples had a shift of the IAC of  $>5 C_T$  values (data not shown).

This multiplex assay was applied with an MPN format in a 2004 survey of Alaskan oysters and the results of real-time PCR compared with culture isolation. Table 4 shows the MPN values generated by both methods; any sample negative for all targets by all methods was omitted. *tlh*-positive, *tdh*-positive, and *trh*-positive *V. parahaemolyticus* bacteria were isolated from nine (33%), five (19%), and seven (26%) of the samples, respectively, by conventional culture methods. The *tlh*, *tdh*, and *trh* genes were detected in 12 (44%), 12 (44%), and 14 (52%),

TABLE 4. Alaskan oyster samples positive for *tlh*, *tdh*, and/or *trh* by real-time multiplex PCR and culture

Sample <sup>b</sup>	MPN value (MPN/g) for target gene in <sup>a</sup> :					
	Real-time PCR			Culture		
	<i>tlh</i>	<i>tdh</i>	<i>trh</i>	<i>tlh</i>	<i>tdh</i>	<i>trh</i>
872	40.5	40.5	40.5	4.05	ND	4.05
873	40.5	40.5	40.5	0.405	ND	0.405
888	4.05	4.05	40.5	4.05	4.05	4.05
889	10.99	10.99	10.99	4.05	4.05	4.05
936	4.05	4.05	4.05	1.099	1.099	1.099
937	4.05	4.05	4.05	0.405	0.405	0.405
1022	0.1099	0.0405	0.405	0.1099	ND	ND
1023	0.0405	0.0405	0.0405	0.0405	0.0405	0.0405
1047	0.405	0.405	0.405	0.405	ND	ND
1048	0.1099	0.0405	0.0405	ND	ND	ND
1054	>0.1099	>0.1099	>0.1099	ND	ND	ND
1072	ND	ND	>1.099	ND	ND	ND
1073	ND	ND	10.99	ND	ND	ND
1229	0.0405	0.0405	0.0405	ND	ND	ND

<sup>a</sup> ND, not detected; limit of detection,  $<0.0405$  MPN/g, MPN value per gram of oyster.

<sup>b</sup> Samples that were negative for all gene targets by both methods are omitted from the table.

respectively, of the 27 oyster samples analyzed by the multiplex assay described here (Table 4). The IAC showed slight to no inhibition in 98% of the MPN tubes analyzed. All but one of the samples in which the IAC showed inhibition were negative for all *V. parahaemolyticus* targets. A comparison between detection by real-time PCR and culture methods was also examined for the 306 MPN tubes analyzed in the 27 samples. Real-time PCR detected *tlh*, *tdh*, and *trh* in 16, 15, and 20%, respectively, of the MPN tubes. In contrast, culture methods detected *tlh*, *tdh*, and *trh* in 7, 5, and 6% of the tubes, respectively. All culture-positive tubes were also PCR positive.

## DISCUSSION

Interest in *V. parahaemolyticus* increased with the emergence of pandemic strains that resulted in outbreaks of unprecedented magnitude (32) and the geographical expansion of *V. parahaemolyticus* illnesses (28). The enumeration of pathogenic *V. parahaemolyticus* bacteria in seafood or the environment is extremely challenging, as the organisms usually occur at low levels in a background of nonpathogenic *V. parahaemolyticus* strains and related species. Molecular methods have been employed in outbreak investigations (10, 12), surveys (8, 9, 14, 30, 41), and ecological studies (13, 17, 20); however, PCR methods for the detection of *V. parahaemolyticus* may not allow the detection of low numbers of pathogenic strains. Additionally, previously described methods (7, 10, 20, 36) do not include an IAC to identify PCR inhibition by seafood and environmental matrices. Therefore, we have developed a real-time PCR multiplex assay which is optimized for the detection of low levels of *tdh* and *trh* in the presence of high levels of total *V. parahaemolyticus* bacteria and which also includes an IAC.

Competition for PCR reagents by individual targets is a concern in any multiplex PCR assay. This is of special concern for the detection of *V. parahaemolyticus* because of the low frequency of pathogenic strains (usually 0.3 to 3%) in the total *V. parahaemolyticus* population that is typical of most seafood and environmental samples (4, 43). We demonstrated that if primers for *tlh*, *tdh*, and *trh* were in equal concentrations, pathogenic strains were detected only when they constituted at least 7% (1:15) of the population. Limiting the *tlh* primer concentration permitted the accurate detection of total *V. parahaemolyticus* bacteria and conserved the remaining PCR components for amplification of the targets (*tdh*, *trh*) present in lower copy numbers. Primer limiting of the *tlh* reaction detected pathogenic strains that constituted only 0.007% (1:15,000) of the *V. parahaemolyticus* population.

The inclusion of an IAC allowed the identification of potential false-negative PCR outcomes resulting from sample matrix inhibition. This feature was not employed in our previous real-time PCR assays (7, 21) or in other PCR (5, 19, 30) or real-time PCR assays reported for *V. parahaemolyticus* (10, 44). There was little PCR inhibition from the Alaskan oysters; only 7 of 306 MPN tubes produced negative results or  $C_T$  values of  $>25$  for the IAC.

The real-time PCR assay for the detection of total and pathogenic *V. parahaemolyticus* bacteria was designed to avoid the amplification of similar sequences listed in GenBank. Regardless, the assay detected the *tdh* gene in some strains of *V.*

*hollisae*, but at a much higher  $C_T$  than for *V. parahaemolyticus*. There is a high degree of homology between the *tdh* genes of *V. parahaemolyticus* and *V. hollisae*, and as both species have a similar ecology, it is possible that both *V. hollisae* and non-pathogenic *V. parahaemolyticus* could be present in the same sample. However, it is unlikely that *V. hollisae* *tdh* would be detected unless it was in high abundance, since the efficiency of our real-time PCR for the *tdh* gene in *V. hollisae* is much less than that for *V. parahaemolyticus*. Public health is protected by an assay that occasionally reports a false positive. In the case of our assay's detecting the *tdh* gene of *V. hollisae*, the false positive would be associated with a well-documented human pathogen associated with oyster consumption.

The multiplex real-time PCR assay is potentially quantitative; however, the limit of detection of the assay (500 CFU/ml of sample) did not provide the desired sensitivity, as we expected few oyster samples to have total or pathogenic *V. parahaemolyticus* levels that high. Therefore, we utilized an MPN-PCR approach to address the challenge of simultaneous detection of total and pathogenic *V. parahaemolyticus* bacteria in Alaskan oysters. MPN analysis estimates total *V. parahaemolyticus* bacteria using colony isolation (11) and adjusts the limit of detection by changing the sample portions. The PCR-MPN approach also reduces the likelihood of detecting dead bacteria due to their dilution in the MPN series before enrichment.

For all samples and MPN dilutions, our multiplex real-time PCR assay detected more tubes positive for total and pathogenic *V. parahaemolyticus* bacteria than standard colony isolation and never failed to detect the appropriate target when *V. parahaemolyticus* was isolated by culture. As expected, the greatest increase in detection by real-time PCR over traditional methods was with pathogenic *V. parahaemolyticus* (as pathogenic isolates are not distinguishable from nonpathogenic isolates on TCBS) and at MPN dilutions containing larger amounts of oyster homogenate (as competing microflora can overgrow TCBS plates). The ratios of pathogenic to total *V. parahaemolyticus* bacteria in Alaskan oysters were previously reported (28) to be much higher than the 0.2 to 3.0% used in the Food and Drug Administration *V. parahaemolyticus* risk assessment (4) and other previous studies (8, 20, 22, 43). As a result, findings for nearly half of the tubes PCR positive for pathogenic *V. parahaemolyticus* were culture confirmed, with a minimal effort of examining up to three suspect colonies from TCBS plates streaked from each turbid APW tube. Failure to culture confirm positive *V. parahaemolyticus* PCR results occurred primarily on TCBS plates that were overgrown with large yellow colonies that were usually *V. alginolyticus* (data not shown).

A potential drawback of applying PCR or real-time PCR to mixed cultures such as APW enrichments is that the target genes might occur in aquatic bacterial species other than *V. parahaemolyticus*. There are countless variants of most *Vibrio* spp. in nature, and relatively few genes from limited strains have been sequenced. During the current study with Alaskan oysters, a target gene that was only reported in *V. parahaemolyticus* was found in a near-neighbor species (16). For 4 of the 27 oyster samples, either *trh* was the only gene detected or it was detected at a greater level than *tlh*. Previously, *trh* was identified only in *V. para-*

*haemolyticus*, and it was unexpected to find it in the absence of *tlh*. A sucrose-positive colony that was subsequently identified as *V. alginolyticus* was the source of the *trh* gene. This finding suggested that, if a *trh*-positive strain of *V. alginolyticus* occurred in the same MPN tube as nonpathogenic *V. parahaemolyticus*, the real-time PCR result could be erroneously interpreted as the presence of pathogenic *V. parahaemolyticus*. However, this real-time PCR assay was applied to oysters from other parts of the country and *trh* was not detected at a higher level than *tlh* (data not shown), indicating that this might be a problem unique to the region.

These results demonstrate that our multiplex real-time PCR assay, when applied in an MPN format, provides a robust and practical means for the detection of a wide range of naturally occurring levels of total and pathogenic *V. parahaemolyticus* bacteria in oysters.

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